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A 96-well single-pot liquid–liquid extraction, hydrophilic interaction liquid chromatography–mass spectrometry method for the determination of muraglitazar in human plasma

Y.-J. Xue *, Jane Liu, Steve Unger

Preclinical Candidate Optimization, Pharmaceutical Research Institute, Bristol-Myers Squibb, New Brunswick, New Jersey 08903, USA

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Abstract

A single-pot liquid–liquid extraction (LLE) with hydrophilic interaction liquid chromatography/tandem mass spectrometry (HILIC/MS/MS) method has been developed and validated for the determination of muraglitazar, a hydrophobic diabetes drug, in human plasma. To 0.050 ml of each plasma sample in a 96-well plate, the internal standard solution in acetonitrile and toluene were added to extract the compound of interest. The plate was vortexed, followed by centrifugation. The organic layer was then directly injected into an LC/MS/MS system. Chromatographic separation was achieved isocratically on a Thermohypersil_Keystone, Hypersil silica column (3 mm × 50 mm, 3 μm). The mobile phase contained 85% of methyl *t*-butyl ether and 15% of 90/10 (v/v) acetonitrile/water with 0.3% trifluoroacetic acid. Post-column mobile phase of 50/50 (v/v) acetonitrile/water containing 0.1% formic acid was added. Detection was by positive ion electrospray tandem mass spectrometry on a Sciex API 4000. The standard curve, ranged from 1 to 1000 ng/ml, was fitted to a $1/x$ weighted quadratic regression model. This single-pot LLE approach effectively eliminated time-consuming organic layer transfer, dry-down, and sample reconstitution steps, which are essential for a conventional liquid–liquid extraction procedure. The modified mobile phase was more compatible with the direct injection of the commonly used extraction solvents in LLE. Furthermore, the modified mobile phase improved the retention of muraglitazar, a hydrophobic compound, on the normal phase silica column. The validation results demonstrated that this method was rugged and suitable for analyzing muraglitazar in human plasma. In comparison with a revised-phase LC/MS/MS method, this single-pot LLE, HILIC/MS/MS method improved the detection sensitivity by more than four-fold based upon the LLOQ signal to noise ratio. This approach may be applied to other hydrophobic compounds with proper modification of the mobile phase compositions.

Keywords: Single-pot; Liquid–liquid extraction; Hydrophilic interaction liquid chromatography; LC/MS/MS; Muraglitazar

1. Introduction

Muraglitazar (Fig. 1) is a novel oxybenzylycine analog that shows potent and balanced agonist activity at both α and γ PPAR (peroxisome proliferator-activated receptor) isoforms [1]. Activation of PPARα results in decreased circulating triglyceride levels and increased HDL cholesterol levels in humans [2]. Activation of PPARγ results in improved insulin sensitivity and glucose utilization [3,4]. Dual agonism of PPARα and PPARγ is a novel monotherapeutic approach for the treatment of type 2 diabetes and its often-associated dyslipidemia. Currently, this compound is being developed for the treatment of type 2 diabetes [1,5].

Reversed-phase high performance liquid chromatography (RP-HPLC) has been widely used in the pharmaceutical industry as a means of chromatographic separation [6]. However, for the separation of very polar compounds, high aqueous mobile phases need to be used in order to achieve desired retention on RP-HPLC columns. The high water content in turn hinders ionization and desolvation of the compound of interest in the ion source of mass spectrometry [7]. The use of partial aqueous mobile phase with a traditional normal phase analytical column has been termed as hydrophilic interaction liquid chromatography (HILIC). Hydrophilic interaction between the compound and the silica stationary phase is the major on-column retention mechanism of the ionized molecules. Therefore, in HILIC, an

* Corresponding author. Tel.: +1 732 227 6330; fax: +1 732 227 3762.
E-mail address: yongjun.xue@bms.com (Y.-J. Xue).

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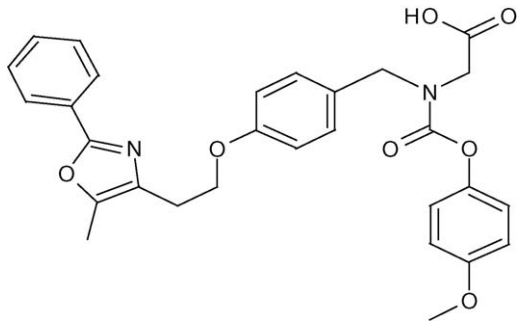

Fig. 1. Chemical structure of muraglitazar.

increase of water content in the mobile phase would result in a decrease of retention time [8]. Due to the unique separation mechanism, HILIC is growing to be an alternative to RP-HPLC for the analysis of polar compounds with LC/MS/MS. In addition to the improved retention, comparing to RP-HPLC for the separation of polar compounds, the higher percentage of organic content allowed in HILIC contributes to more efficient desolvation and ionization in mass spectrometer [8], which can lead to improved sensitivity.

There are numerous reports on successful combinations of HILIC/MS/MS with three commonly used sample clean-up procedures (protein precipitation, liquid–liquid extraction and solid phase extraction) in bioanalytical analysis. A simple protein precipitation procedure in conjunction with HILIC/MS/MS has been successfully applied to measure several drug candidates in biological matrices [9–12]. For solid phase extraction (SPE), there are reports on using highly automated 96-well SPE with HILIC/MS/MS for the determination of drug candidates in plasma [13–17]. Weng reported the direct injection of reversed-phase solid phase extracts onto a silica column for the determination of a range of polar pharmaceutical compounds in biological fluids [18]. For liquid–liquid extraction (LLE), there are reports on LLE in conjunction with HILIC/MS/MS for analyzing biological samples, where sample extracts were dried down and reconstituted before the injection [13,20–26]. More recently, a direct injection LLE approach was used for the analysis of polar compounds, such as isoniazid and cetirizine, in animal and human plasma [19]. Weng recently reported the direct injection of 96-well organic extracts onto a silica column for the determination of several polar compounds in human plasma [27]. A 0.05 ml aliquot of plasma was extracted into 1 ml of methyl *t*-butyl ether or 0.5 ml of ethyl acetate.

So far, there has been no report on using HILIC/MS/MS for the determination of hydrophobic compounds. Common understanding is that the RP-LC/MS/MS is more suitable for this class of compounds, since hydrophobic compounds are usually well retained on a reverse phase column. Because of the superior sensitivity gain demonstrated for polar compounds using HILIC/MS/MS, exploring and optimizing conditions for analysis of hydrophobic compounds is important.

The objective of this work was to develop a single-pot LLE, HILIC/MS/MS method for the determination of muraglitazar, a hydrophobic diabetes drug, in human plasma. In this method, a small volume of toluene (0.20 ml) and acetonitrile (0.05 ml) was used to extract muraglitazar from 0.05 ml of human plasma samples in 96-well plate format. The top organic layers were then directly injected into the LC/MS/MS system, where a silica column and toluene miscible mobile phase, 85% of methyl *t*-butyl ether and 15% of 90/10 (v/v) acetonitrile/water with 0.3% trifluoroacetic acid, were used for muraglitazar separation under HILIC conditions. A full validation was performed to assess the accuracy, precision, linearity, and the lower limit of quantitation of the method. The results presented here demonstrate that this single-pot LLE, HILIC LC/MS/MS method is feasible for analyzing muraglitazar in human plasma. In comparison with a RP-LC/MS/MS method for muraglitazar, HILIC/MS/MS showed more than four-fold of improvement in signal to noise ratio.

2. Experimental

2.1. Reagents and chemicals

Muraglitazar (Fig. 1) and its internal standard (IS) were provided by the Analytical Research and Development, Bristol-Myers Squibb Pharmaceutical Research Institute [1]. The pK_a of the carboxylic acid moiety in muraglitazar was experimentally determined to be 3.6. The internal standard was a closely related structural analog of muraglitazar. However, due to proprietary reasons, its chemical structure cannot be shown. Acetonitrile (HPLC grade), trifluoroacetic acid (97%, TFA), and formic acid (98%, GR) were purchased from EM Science (Gibbstown, NJ, USA). Toluene and methyl *t*-butyl ether were also purchased from EM Science. In-house deionized water, further purified with a Milli-Q water purifying system (Millipore Corporation, Bedford, MA, USA), was used. Drug-free human plasma was purchased from Lampire Biological Laboratories (Pipersville, PA, USA).

2.2. Chromatographic conditions

For HILIC separation, muraglitazar and its IS were separated isocratically with 85% of methyl *t*-butyl ether and 15% of 90/10 (v/v) acetonitrile/water containing 0.3% trifluoroacetic acid. The flow rate was 0.3 ml/min at room temperature. The separation column was on a Thermohypersil_Keystone, Hypersil silica column (3 mm × 50 mm, 3 μm, Bellefonte, PA). Post-column addition of mobile phase was 50/50 (v/v) acetonitrile/water containing 0.1% formic acid at the flow rate of 0.15 ml/min. The injection volume was 3 μl and the run time was 2.0 min.

For RP-HPLC separation, muraglitazar and its IS were separated isocratically, using 80/20 (v/v) of acetonitrile/water containing 1 mM formic acid. The flow rate was 0.3 ml/min at room temperature. The separation column was a Phenomenex Luna C18 (2) analytical column (2 mm × 50 mm, 5 μm, Torrance, CA). The injection volume was 10 μl and the run time was 2.0 min. The similar RP-HPLC separation conditions were reported before, where 0.1% formic acid was used for mobile phase preparation [28].

2.3. Instrumentation

The liquid chromatography separation system used for both HILIC/MS/MS and RP-HPLC/MS/MS methods consisted of three Shimadzu LC-10AD pumps (Columbia, MD, USA) and a Perkin-Elmer Series 200 LC autosampler (Norwalk, CT, USA).

A Sciex API 4000 LC–MS/MS system (Foster city, CA, USA) operating under Analyst v1.1 software was used for both HILIC/MS/MS and RP-LC/MS/MS methods. The electrospray ion source was run in a positive ionization mode for all experiments. The typical ion source parameters were: capillary voltage 4200 kV, declustering potential (DP) 61 V, entrance potential (EP) −10 V, collision energy (CE) 35 V, collision cell exit potential (CXP) 14 V, deflector −100 V, channel electron multiplier (CEM) 2500 V, source temperature 325 °C. Nebuliser gas (NEB), Curtain gas (CUR), and collision gas (CAD) were set to 10, 12 and 6 of the state file parameters, respectively. Nitrogen gas was used for CUR, CAD, and NEB. The samples were analyzed via selected reaction monitoring (SRM). The monitoring ions were set to m/z 517 → 186 for muraglitazar and m/z 531 → 306 for its IS. The scan dwell time was set 0.15 s for both channels. The similar RP-LC/MS/MS method published earlier was conducted on a Sciex API 3000 [28].

2.4. Standard, quality control and internal standard preparations

The standard and quality control preparation procedures were reported before [28]. Briefly, a 1 mg/ml standard stock solution in acetonitrile was used to prepare the standard curves: 1, 2, 12.5, 25, 50, 250, 500, 750, and 1000 ng/ml. A 1 mg/ml quality control (QC) stock solution was used for QC preparation: 3, 400, 800, and 16,000 ng/ml. A 1 mg/ml internal standard (IS) stock solution was used to prepare the 50 ng/ml working internal standard solution in acetonitrile with 0.1% formic acid.

The same standards, QCs and IS were used for either HILIC/MS/MS or RP-LC/MS/MS method.

2.5. Sample processing procedure

2.5.1. HILIC/MS/MS

To 0.05 ml of each standard or QC sample in a microtube, 0.05 ml of the working IS solution and 0.2 ml of toluene were added. The microtubes were capped, shaken for 2 min, and then centrifuged for 10 min at 3200 rpm. The top organic layers were injected directly into the LC/MS/MS system.

2.5.2. RP-LC/MS/MS

To 0.10 ml of each standard and QC sample in a microtube, 0.4 ml of the working IS solution was added. The tubes were capped, vortexed for 2 min, and then centrifuged for 10 min at 3200 rpm. The supernatant layers were directly injected into the LC/MS/MS system.

3. Results and discussion

3.1. Method optimization

3.1.1. Sample preparation for the single-pot LLE

In a traditional liquid–liquid extraction method, human plasma samples initially are extracted with large volume of organic solvents, such as toluene or methyl *t*-butyl ether. The organic layers then have to be transferred, dried down, and the residues are reconstituted into the mobile phase for analysis. Those steps are time consuming and can potentially cause sample loss due to incomplete organic layer transfer and compound adsorption onto container walls. Furthermore, the excessive heat applied during the dry-down step could cause degradation of heat labile compounds [27].

There are major challenges in developing a single-pot LLE method. These include effective elimination of the irregular emulsion caused when using a small volume of organic solvent and consistently obtaining high recovery.

During the early stage of the experiment, the effect of acetonitrile level on LLE was investigated at selected toluene volumes (0.1–0.3 ml). In the absence of acetonitrile or when its volume was less than 0.05 ml, irregular emulsion formed between the organic and aqueous layers during shaking. Even high speed centrifugation could not break the irregular emulsion, and no clear aqueous-organic boundary was formed for most of the samples tested. This phenomenon was consistent with our earlier observation with methyl *t*-butyl ether as the extraction solvent [29]. In the end, 0.05 ml of acetonitrile was selected for the extraction of 0.05 ml plasma. In addition, 0.1% formic acid was added to acetonitrile to lower the plasma pH to ensure that muraglitazar stayed as the non-ionized form ($pK_a = 3.6$), which could be readily extracted by toluene [30].

With the plasma and acetonitrile volume being defined as 0.05 ml each, the impact of toluene volume to the extraction efficiency was investigated by varying toluene volume for the extraction. The results indicated that satisfactory extraction efficiency was obtained for toluene volume ranging from 0.05 to 0.3 ml. This meant that even 0.05 ml of toluene would be sufficient for the extraction of 0.05 ml of plasma along with 0.05 ml of acetonitrile. This single-pot LLE could be scaled up to large human plasma volumes. For a successful scale-up, same or similar volume of plasma and acetonitrile should be used, and the toluene to plasma ratio should be kept between 1 and 6.

To compare the single-pot LLE with the dry-down LLE procedure, 0.60 ml of toluene was used for the dry-down procedure for an easier and more complete transfer of the organic layer, and 0.20 ml of toluene was used for the single-pot LLE. In both cases, 0.05 ml plasma and the same volume of acetonitrile with 0.1% formic acid were used. The experiment results showed that the recovery for the dry-down procedure was about 25% lower than that of the single-pot LLE procedure. One key contributing factor for the lower recovery of the dry-down procedure was the incomplete organic layer transfer, along with some loss during the dry-down and reconstitution steps. This suggested that the single-pot LLE procedure could be more efficient than the traditional dry-down LLE procedure.

In comparison with the previous work [27], there were two major improvements for this single LLE procedure. First, toluene was used instead of methyl *t*-butyl ether, so the evaporation was reduced during sample injection. Second, no sample dilution was involved for this single-pot LLE, when 0.05 ml of toluene was used for extracting 0.05 ml human plasma. However, there was significant sample dilution for the previous work [27], where at least 1 ml methyl *t*-butyl ether or 0.5 ml ethyl acetate was used to extract 0.05 ml of human plasma.

3.1.2. LC conditions for HILIC/MS/MS

For most of HILIC/MS/MS methods reported so far, a composition of water and acetonitrile was used as the mobile phase which was compatible with mass spectrometry in term of ionization and desolvation [8]. However, such a mobile phase system was not compatible with most of LLE extracts for the direct injection into an HILIC/MS/MS system. A mismatch between the mobile phase and the LLE extracts can cause peak broadening or distortion [8]. To alleviate this problem, methyl *t*-butyl ether was added to the acetonitrile/water mobile phase. The modified mobile phase (methyl *t*-butyl ether/acetonitrile/water) was more compatible with organic solvents used for LLE [8,27]. As a result of the mobile phase modification, toluene could be used for muraglitazar extraction.

The modified mobile phase was a much weaker elution solvent system in the HILIC mode; therefore, muraglitazar, a hydrophobic compound, could be retained on a bare silica column. Without the addition of methyl *t*-butyl ether, muraglitazar would elute at the solvent front. There were two major differences in comparison with the regular HILIC methods reported so far [8–26]. First, with 15% of 90/10 (v/v) acetonitrile/water being used in the modified mobile phase, the actual content of water used in the mobile phase was only 1.5%, which was less than 5% suggested for HILIC methods. Secondly, the weak elution solvents were no longer limited to acetonitrile and methanol, which further extended HILIC separations to less polar compounds and reduced the distinction between HILIC and normal phase. In addition, two mobile phases (methyl *t*-butyl ether and 90/10 (v/v) acetonitrile/water) were miscible over the entire range of composition.

Buffer systems would have strong influence in the retention mechanism. Several buffer systems were investigated with trifluoroacetic acid providing the best peak shape. It was believed that at the strong acidic condition, hydrophilic interaction appeared to be the dominate force for the on-column retention of muraglitazar. Under the optimized HILIC separation conditions (85% of methyl *t*-butyl ether and 15% of 90/10 (v/v) acetonitrile/water containing 0.3% trifluoroacetic acid), the retention times of muraglitazar and the internal standard were 0.94 and 0.92 min and the chromatographic run time was 2 minutes (Figs. 2b and 4b). The less polar IS was eluted first, as predicted.

With a carboxylic acid functional group, muraglitazar forms acyl glucuronide, which was prone to convert back to the parent in the ion source under normal mass spectrometry operation conditions. Therefore, chromatographic separation between muraglitazar and its acyl glucuronide was required for the accurate measurement of the parent in human plasma. A sample containing acyl glucuronide was injected into LC/MS/MS under HILIC separation conditions, and the chromatogram obtained demonstrated that acyl glucuronide was well separated from the parent peak with the retention time of 1.40 min.

One drawback with the inclusion of methyl *t*-butyl ether in the mobile phase was that the modified mobile phase was less compatible with the mass spectrometry. So a third HPLC pump was used to enhance electrospray response. During the experiment, we observed that the flow rate and the compositions of the post-column solvents could dramatically influence sensitivity and peak shape. Based on the preliminary experiment results, it was found that 0.15 ml/min of 50/50 acetonitrile/water (v/v) containing 0.1% formic acid was the best for this method. The post-column additives were fully miscible with the mobile phase, and did not affect chromatographic resolution [31,32]. Although trifluoroacetic acid was known to suppress the electrospray signals of anatytes due to its ability to form gas-phase ion pairs with positively charged analyte ions [12,17], the post-column addition of formic acid along with acetonitrile in this HILIC method apparently alleviated such suppression exerted to muraglitazar and its IS by trifluoroacetic acid mobile phase.

Several different brands of silica columns were investigated in term of the peak shape and retention time for the compounds of interest. Among these columns tested, Thermohypersil_Keystone, Hypersil silica column appeared to be superior over others, such as Phenomenex Luna silica column and Waters HILIC column, in terms of peak shape for the analyte; therefore, it was selected for further method development and validation.

A RP-LC/MS/MS method was used for comparison. The RP-LC method employed 80% of acentonitrile and 20% buffer solution on a Luna C18 (2) column (Section 2.2) at similar retention times.

3.2. Mass spectrometry

A Sciex API 4000 mass spectrometer was used to monitor muraglitazar and its internal standard in the extracted samples. The MS spectra for both compounds were dominated by the $[M+H]^+$ ions: *m/z* 517 for muraglitazar and *m/z* 531 for IS. The MS/MS product ion spectra of the $[M+H]^+$ for both compounds produced major product ions at *m/z* 186 and 306, respectively. Thus, the SRM used was *m/z* 516 → 186 for muraglitazar and *m/z* 531 → 306 for the internal standard. Both the analyte and the IS produced the common product ion: *m/z* 186. However, when the quantitative optimization (auto-tune) was performed on the API 4000, a different product ion, *m/z* 306, was chosen by Analyst software for the IS. The same mass spectrometry conditions were used for both HILIC and RP-HPLC method validations.

3.3. Method validation

For the validation of the single-pot LLE method, 0.2 ml of toluene and 0.05 ml of acetonitrile were used to extract 0.05 ml of human plasma. Similarly, 0.4 ml of acetonitrile was used to extract 0.1 ml human plasma. As a result, an identi-

Table 1
Individual standard curve concentration data for muraglitazar in human plasma for HILIC/MS/MS

Spiked concentration	Run 1		Run 2		Run 3	
	Predicted concentration	% Dev	Predicted concentration	% Dev	Predicted concentration	% Dev
1.00	0.89	−11.0	0.88	−12.0	0.99	−1.0
1.00	1.12	12.0	0.85	−15.0	1.04	4.0
2.00	2.03	1.5	2.20	10.0	1.96	−2.0
2.00	1.78	−11.0	1.97	−1.5	1.70	−15.0
12.5	13.10	4.8	15.91	27.3	13.42	7.4
12.5	13.00	4.0	12.72	1.8	11.80	−5.6
25.0	21.40	−14.4	24.81	−0.8	28.25	13.0
25.0	25.42	1.7	22.69	−9.2	25.72	2.9
50.0	51.21	2.4	52.14	4.3	51.27	2.5
50.0	55.87	11.7	44.61	−10.8	47.22	−5.6
250	286.42[a]		252.25	0.9	261.93	4.8
250	245.76	−1.7	286.24	14.5	242.37	−3.1
500	519.89	4.0	514.70	2.9	508.46	1.7
500	491.22	−1.8	446.20	−10.8	494.10	−1.2
750	731.30	−2.5	746.04	−0.5	706.81	−5.8
750	737.88	−1.6	716.54	−4.5	753.15	0.4
1000	1038.72	3.9	1066.10	6.6	1049.80	5.0
1000	981.10	−1.9	976.63	−2.3	988.06	−1.2

Concentration: ng/ml.
[a] Studentized residual outliers.

cal dilution factor was for both methods for a better direct comparison.

3.3.1. Standard curves

After the single-pot LLE, HILIC/MS/MS conditions were defined, a full validation was performed to assess the performance of the method. A nine-point calibration standard curve ranging from 1 to 1000 ng/ml of muraglitazar in human plasma was used in duplicate in each analytical run. Peak area ratios of muraglitazar to IS were used for regression analysis. Both linear and quadratic regression models were evaluated. However, the quadratic model provided better curve fitting due to the wide calibration range. Therefore, the weighted ($1/x$) quadratic regression model, where x is the concentration of muraglitazar, was used for this validation. Table 1 shows the summary of the individual standard curve data obtained in the three runs used to determine the accuracy and precision of the single-pot LLE, HILIC/MS/MS method. All but one of the 54 standards in the three analytical runs gave back calculated concentrations within 15.0% of their spiked concentrations. The regression coefficients (R-squared) for the three runs were greater than 0.996. For the RP-LC/MS/MS method, among the 72 standards in the four separate runs, three of them were rejected due to preparation errors, and one had % deviation greater than 15% of its spiked value. It was evident that standards behaved similarly under both HILIC/MS/MS and RP-LC/MS/MS conditions, although the RP-LC/MS/MS method apparently gave slightly better accuracy with percent deviations (% DEV) within 12.0%. But in both cases, standards were well within the requirements set forth by the FDA guidance [33]. Therefore, it was concluded that the calibration curves used in this single-pot LLE, HILIC/MS/MS method were precise and accurate for the measurement of muraglitazar in human plasma.

3.3.2. Accuracy and precision

The accuracy and precision of the method was assessed by analyzing the low, medium and high QC samples (3, 400 and 800 ng/ml). A dilution QC sample (16,000 ng/ml), with a concentration higher than the upper limit of the standard curve range, was also analyzed. This QC sample was diluted 1:19 with control human plasma prior to processing and analysis, so that it would fall within the calibration range. Five replicate samples at each concentration were analyzed in three separate runs. Accuracy was determined by calculating the deviations of the predicted concentrations from their spiked values. The intra- and inter-day precision was expressed as percent coefficient of variation (% CV). Table 2 shows the summary of the individual QC data obtained in the three runs used for the single-pot LLE, HILIC/MS/MS method validation. The deviations of the predicted concentrations from their spiked values were within ±15% for 92% of 60 QC samples. To further assess the accuracy and precision, a one-way analysis of variance (ANOVA) was performed for the three runs, and the results are shown in Table 3. The intra-day precision ranged from 3.8 to 9.6% CV and the inter-day precision ranged from 2.6 to 5.1% CV. The assay accuracy was within ±8.3% of the spiked values. For the RP-LC/MS/MS method validation, the deviations were within 13.3% for all of 80 QC samples. The intra-day precision ranged from 2.0 to 4.7% CV and the inter-day precision ranged from 2.3 to 4.3% CV. The assay accuracy was within ±4.7% of the spiked values. Therefore, the QC validation data from the single-pot LLE, HILIC/MS/MS method were comparable with that of the RP-LC/MS/MS one. Based on the above results, it can be concluded that the single-pot LLE, HILIC/MS/MS method was accurate and precise in the determination of muraglitazar concentrations in human plasma. The results from the dilution QC samples demonstrated that even samples with concentrations greater than the upper limit of the standard curve could

Table 2
Individual quality control sample concentration data for muraglitazar in human plasma for HILIC/MS/MS

Spiked concentration	Run 1		Run 2		Run 3	
	Predicted concentration	% Dev	Predicted concentration	% Dev	Predicted concentration	% Dev
3.00	3.40	13.3	2.93	−2.3	3.20	6.7
3.00	3.02	0.7	2.61	−13.0	3.22	7.3
3.00	2.50	−16.7	2.83	−5.7	2.87	−4.3
3.00	3.42	14.0	2.68	−10.7	2.96	−1.3
3.00	2.57	−14.3	2.95	−1.7	3.22	7.3
400	356.20	−11.0	449.09	12.3	348.85	−12.8
400	381.02	−4.7	401.48	0.4	298.46	−25.4
400	367.10	−8.2	410.70	2.7	359.69	−10.1
400	432.32	8.1	380.35	−4.9	393.48	−1.6
400	408.63	2.2	338.46	−15.4	371.11	−7.2
800	747.70	−6.5	768.28	−4.0	850.46	6.3
800	753.39	−5.8	838.52	4.8	803.12	0.4
800	774.22	−3.2	852.19	6.5	826.45	3.3
800	776.03	−3.0	775.28	−3.1	826.34	3.3
800	828.75	3.6	832.58	4.1	841.64	5.2
16000	14336.17	−10.4	14403.59	−10.0	14269.20	−10.8
16000	14919.27	−6.8	15047.68	−6.0	13979.36	−12.6
16000	16324.53	2.0	15008.68	−6.2	12319.29	−23.0
16000	15189.14	−5.1	16300.94	1.9	13805.59	−13.7
16000	16989.71	6.2	12733.45	−20.4	14404.98	−10.0

Concentration: ng/ml.

Table 3
Accuracy and precision for muraglitazar in human plasma for HILIC/MS/MS

	Spiked concentration			
	3.00	400	800	16000
Mean observed concentration	2.96	379.80	806.33	14668.77
% Dev	−1.3	−5.1	0.8	−8.3
Inter-day precision (%CV)	2.6	4.1	2.9	5.1
Intra-day precision (%CV)	9.6	9.5	3.8	7.4
Total variation (%CV)	10.0	10.3	4.8	9.0
n	15	15	15	15
Number of runs	3	3	3	3

Concentration: ng/ml.

be analyzed to obtain acceptable data after dilution with control human plasma.

3.3.3. Lower limit of quantitation (LLOQ)

To establish the LLOQ for the single-pot LLE, HILIC/MS/MS method, six different lots of control human plasma were spiked at 1 ng/ml to obtain six LLOQ samples. The LLOQ samples were processed and analyzed with a standard curve and QC samples, and their predicted concentrations determined. The results of the LLOQ determinations are shown in Table 4. The deviations of the predicted concentrations for five out of six LLOQ samples were within ±18.0% of the spiked value. A typical SRM chromatogram at the LLOQ is shown in Fig. 2b. These results indicated that there was no significant lot-to-lot variation in matrix effect. A typical SRM chromatogram at LLOQ from RP-LC/MS/MS is also shown in Fig. 3b. It is apparent that the single-pot LLE, HILIC/MS/MS method improved the signal to noise ratio of the LLOQ by

Table 4
Lower limit of quantitation determination of muraglitazar in human plasma

Spiked concentration	Predicted concentration	% Dev	Mean concentration	Mean % Dev
1.00	1.26	26.0	1.065	6.5
1.00	1.18	18.0		
1.00	0.93	−7.0		
1.00	0.97	−3.0		
1.00	1.08	8.0		
1.00	0.97	−3.0		

Concentration: ng/ml.

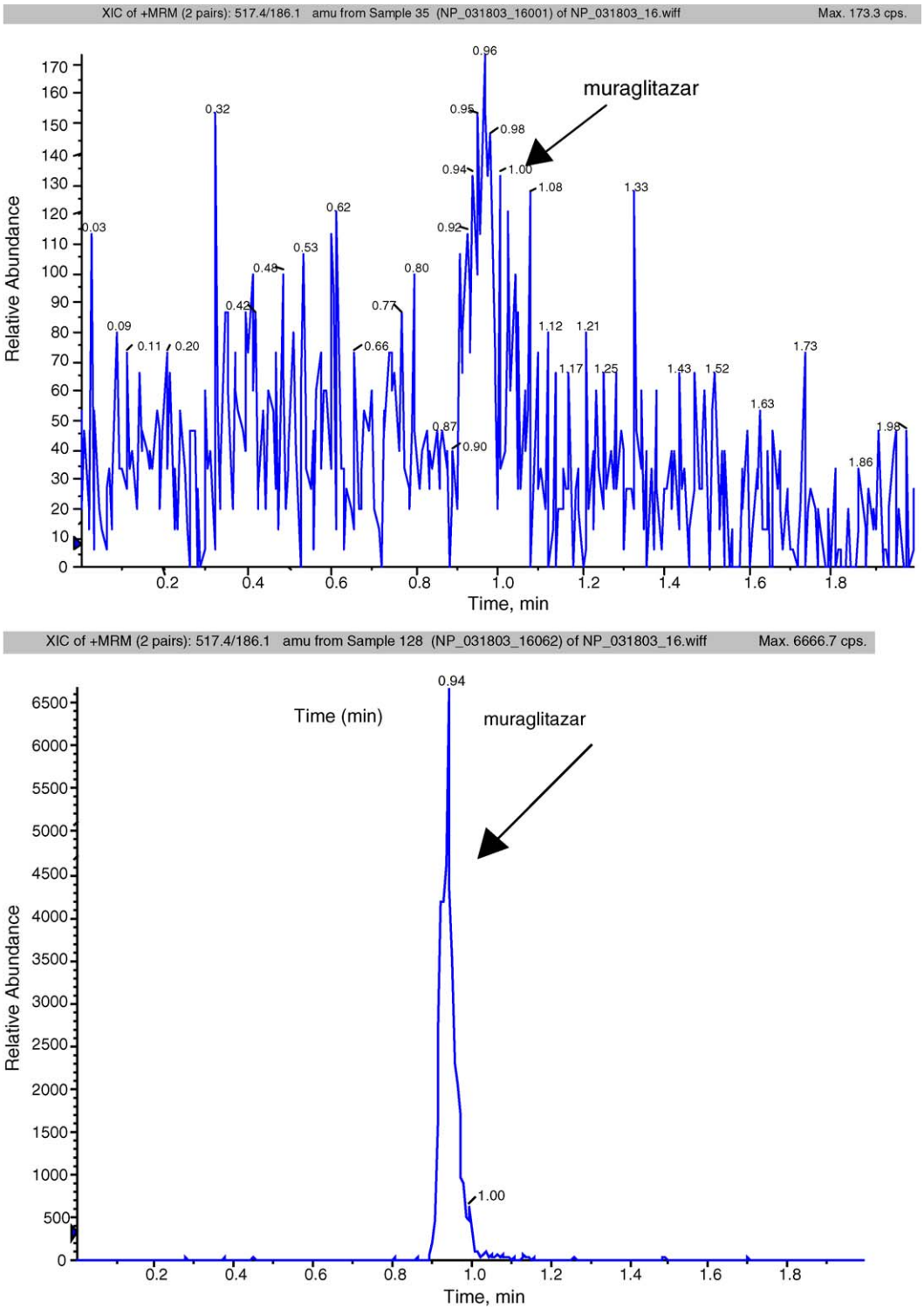

Fig. 2. Selected reaction monitoring chromatograms for muraglitazar from HILIC/MS/MS: (a) blank human plasma (top); (b) human plasma containing muraglitazar at lower limit of quantitation (1 ng/mL) and its internal standard at 50 ng/mL (bottom).

more than four-fold in comparison with the RP-LC/MS/MS one.

There were two possible causes for the improved sensitivity. First, for the HILIC/MS/MS method, the weaker extraction solvent, toluene, was used, and the samples concentrated on the analytical column by the stronger mobile phase. The stronger extraction solvents (0.1% formic acid in the extracts versus 1 mM formic acid in the mobile phase) were used for the RP-LC/MS/MS method. It was evident that the mean FWHM and peak width at the base in the HILIC method was only a half of the RP-LC one (Figs. 2b and 3b). Second, for the HILIC method, the mobile phase was more volatile than that

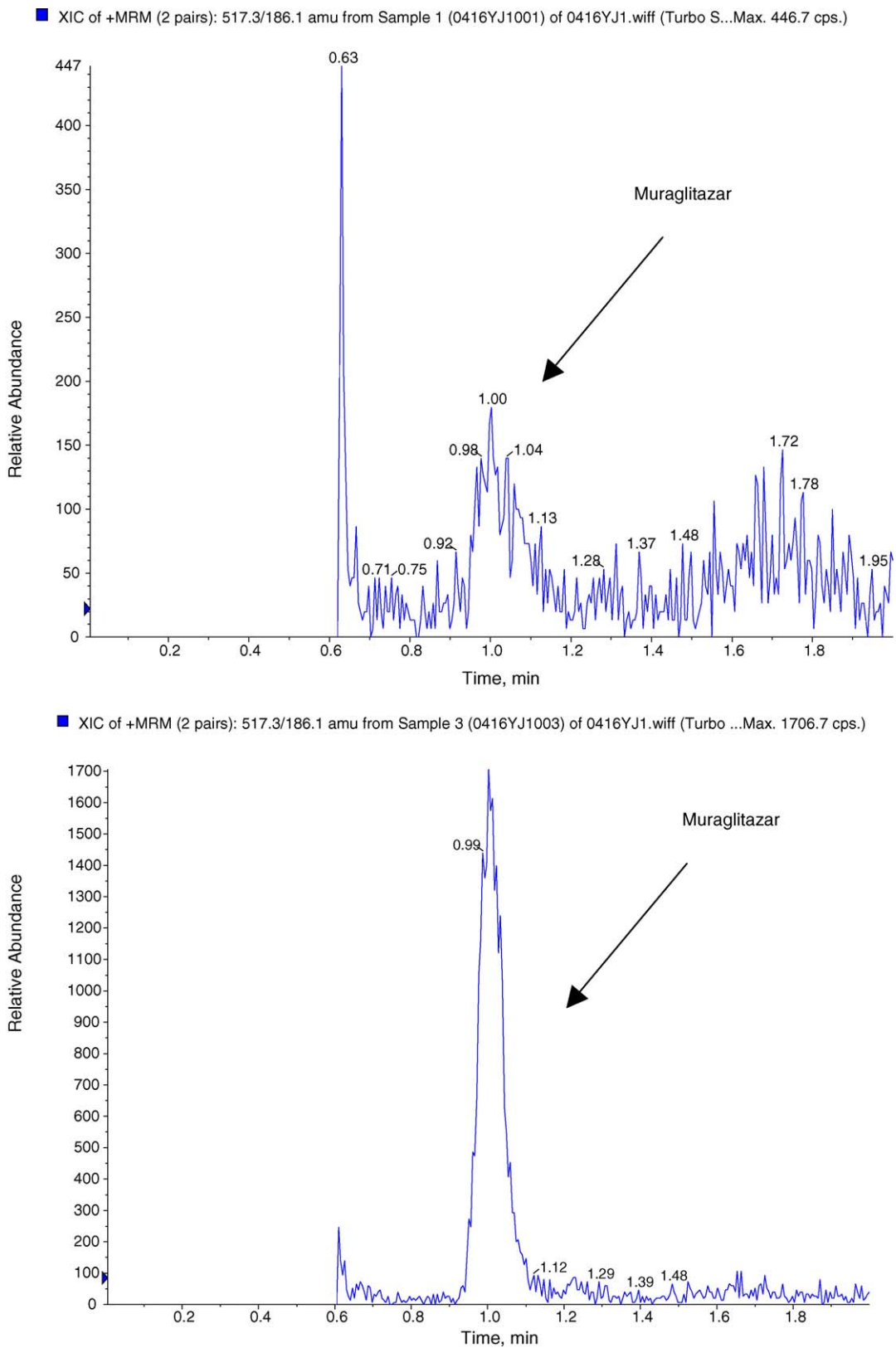

Fig. 3. Selected reaction monitoring chromatograms for muraglitazar obtained from RP-LC/MS/MS: (a) blank human plasma (top); (b) human plasma containing muraglitazar at lower limit of quantitation (1 ng/ml) and its internal standard at 50 ng/ml (bottom).

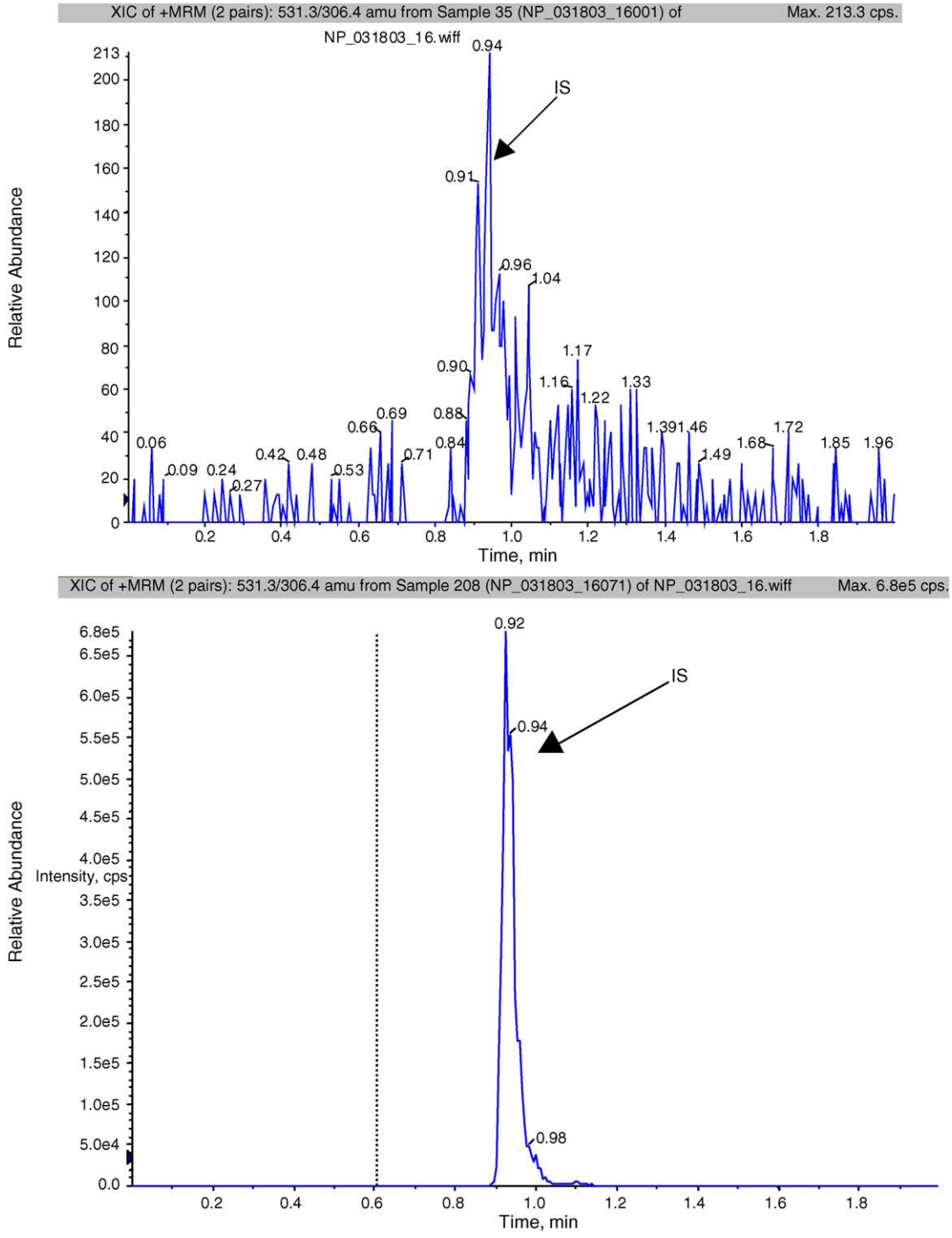

Fig. 4. Selected reaction monitoring chromatograms for the internal standard of muraglitazar obtained from HILIC/MS/MS: (a) blank human plasma (top); (b) human plasma containing only the internal standard at 50 ng/ml (bottom).

used in the RP-LC method, affording more efficient evaporation. For the HILIC method validation, a smaller injection volume (3 μl) was used to achieve the similar peak response for the analyte. If the comparable volume of samples were injected (10 μl instead of 3 μl), the total sensitivity for the single-pot LLE, HILIC/MS/MS method could potentially be further improved [8,13]. Therefore, the use of HILIC/MS/MS improved the method sensitivity. Furthermore, the plasma volume used for HILIC/MS/MS was only 0.05 ml. If higher sensitivity was desired for the method, increasing the sample volume while maintaining the same toluene volume would be possible. In this work, the similar ratio of plasma and acetonitrile was always required to avoid the formation of the irregular emulsion.

3.3.4. Specificity and matrix effect

The use of a structural analog of IS required careful examination of the matrix effect. However, these two compounds were nearly co-eluted under the chromatographic conditions used (Figs. 2b and 4b) and experience the same effect caused by plasma matrix. To confirm this assumption, six different lots of control human plasma were analyzed with and without IS. The degree of interference was assessed by inspection of SRM chromatograms. No significant interfering peaks from the plasma were found at the retention time and in the ion channel of either the analyte or the IS. Figs. 2a and 4a illustrate chromatograms of blank plasma. As a comparison, the SRM chromatogram of blank plasma from the RP-LC/MS/MS is shown in Fig. 3a. It is evidence that the background levels from both methods were very similar, approximately 200 counts/s.

Since this was a single-pot LLE method, the apparent matrix effect (the sum of recovery and matrix effect) was assessed by comparing the average peak areas of five replicates of the neat solution with these of the lowest standard in plasma. The average peak areas of the lowest standard versus that obtained from the corresponding neat solution were 1.19 for the analyte, which indicated that there was approximately 19% enhancement for this method. Combined with the fact that there was no significant lot-to-lot variation in LLOQ and specificity results, it was concluded that such a low matrix effect did not compromise the performance of the method.

4. Conclusions

We have demonstrated a method for the determination of muraglitazar in human plasma by using 96-well single-pot liquid–liquid extraction and HILIC/MS/MS analysis. This single-pot liquid–liquid extraction approach effectively eliminated three time-consuming steps, organic layer transfer, dry-down, and reconstitution, all of which are required by traditional liquid–liquid extraction. In addition, we demonstrated that direct-injection of LLE extracts increased the signal to noise ratio of the LLOQ by more than four-fold. When adjusted for injection volume, the further improvement was expected. Because very small volume of organic solvent was used for the extraction, the sample dilution was very minimal. Furthermore, we have modified the commonly used hydrophilic interaction chromatographic mobile phase system (acetonitrile-water) by adding methyl *t*-butyl ether to the mixture, so that many LLE extracts were now compatible with the mobile phase for direct injection. In this validation, toluene was used as the extraction solvent. The validation results demonstrated that this method was rugged, precise and accurate. Our experience showed that this approach can be used for other hydrophobic compounds with minor modification of the mobile phase. Therefore, the addition of methyl *t*-butyl ether expanded HILIC/MS/MS from polar compounds to more hydrophobic ones which are traditionally separated by RP-HPLC.

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